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Voltage-Gated Calcium Channel $\alpha_2\delta$ Subunits in Lipid Rafts: The Importance of Proteolytic Cleavage Into α_2 and δ

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The High Voltage-Activated (HVA) subgroup of voltage-gated calcium channels contain an $\alpha1$ subunit, which forms the selective pore and determines the main functional properties of the channel. The $\alpha1$ subunit is associated with auxiliary subunits including β and $\alpha2\delta$, which modulate trafficking and functional properties of the channels.

There are four known genes encoding $\alpha2\delta$ subunits, which are believed to have similar structure. They consist of two peptides: the highly glycosylated $\alpha2$ which is entirely extracellular is disulfide-bonded to a δ subunit that links the protein into the plasma membrane. The $\alpha2$ and δ peptides are encoded by a single gene as an uninterrupted $\alpha2\delta$ pre-protein, which is further processed post-translationally. We have recently shown that $\alpha_2\delta$ subunits are glycosyl phophatidyl inositol (GPI)-achored proteins (Davies et al., 2010, PNAS 107:1654-1659), and this is essential for their function, and explains their localization in lipid raft fractions (Davies et al, 2006, J. Neurosci. 26: 8748-8757).

We further studied the mechnism and consequences of the proteolytic cleavage of the $\alpha_2\delta$ subunits ($\alpha_2\delta$-1, -2 and -3)and identified the cleavage sites in $\alpha_2\delta$-2 and $\alpha_2\delta$-3. Western blots from brain tissue revealed only the mature form of the protein strongly associated with lipid rafts. However, transfecting mammalian cells with cDNA for $\alpha2\delta$-1,-2,and -3 resulted in incomplete cleavage (~ 40-60% processing) most probably due to limitations of the cleavage-mediating protease(s) in heterologous expression systems. The mature form is localized in lipid rafts, suggesting that maturation of the protein might occur in localized membrane domains. Moreover, electrophysiological recordings demonstrated that proteolytic cleavage is key to the function of these subunits.

We are currently examining the nature of the protease(s) involved in this proteolytic processing.

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Calcium Dependent Inactivation of $Ca_V1.1$ Channels in Adult Skeletal Muscle: A Possible Role of RyR1 Channels

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Ca2+ dependent inactivation (CDI) has been documented for the great majority of voltage dependent Ca2+ channels (Cav) but whether native Cav1.1 of adult skeletal muscle exhibits this feature remains controversial. When explored in heterologous (i.e., HEK cells) or homologous expression (i.e., myotubes), systems that lack or display immature E-C coupling, respectively, several groups have demonstrated both the absence and presence of Cav1.1 CDI. In a recent report using homologous expression system where CDI was documented, Cav1.1 channels displayed the classical features of CDI: 1) dependence on extracellular Ca2+, 2) elimination by replacing Ca2+ with Ba2+ or by using high affinity and fast kinetics Ca2+ buffer BAPTA, and 3) calmodulin modulation. Here we use whole-cell voltage clamp Ca2+ current recordings obtained from adult skeletal muscle fibers to address whether Cav1.1 exhibits CDI in its native environment. Surprisingly, Ca2+ current inactivation was insensitive to Ca2+ substitution by Ba2+, suggesting that 1) CDI is not present or 2) that Ca2+ flowing through the channels is not the Ca2+ source for current inactivation. We tested the hypothesis that Ca2+ release from SR via ortograde Cav1.1-RyR signaling may be the source for CDI. To test this hypothesis we use dantrolene (a membrane permeable RyR Ca2+ release channel antagonist). When 40 μM dantrolene (a concentration that suppress action potential evoked-Ca2+ transient by ~30%) was added to the external solution Cav1.1 CDI was partially suppressed. These observations suggest the provocative idea that Cav1.1 CDI is regulated by Ca2+ release via adjacent sarcoplasmic reticulum RyR1 channels. Supported by NIH-NIAMS (R01-AR055099).

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CaBP1 and Calmodulin Compete in Regulating Calcium-Dependent Inactivation of CaV1.2

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CaV1.2 is an L-type calcium channel from a family of voltage dependent calcium channels (VDCC) distributed mainly in cardiac and smooth muscle, endocrine cells and neurons, which produce calcium influx in response to membrane depolarization. Interaction of calcium binding protein 1 (CaBP1) and calmodulin (CaM) with the C-terminus (CT) of the L-type CaV1.2 channel is crucial for calcium-dependent inactivation (CDI); CaBP1 has an opposite effect on VDCCs compared to CaM: while CaM promotes CDI, CaBP1 prolongs and facilitates calcium currents and does not support CDI. CaBP1 and CaM are also bind to the N-terminus (NT), but the role of this interactions and the effect of these proteins on each other are unknown. We characterized the interaction of CaBP1 with NT peptides of alpha-1C using pull down assay. This binding site of CaBP1 is between a.a. 95-120 and does not overlap the CaM binding site; the binding is not calcium dependent. Coexpression of CaBP1 with CaV1.2 in Xenopus oocytes eliminated the CaM-dependent CDI. Inversely, titrated expression of fluorescently tagged CaM and CaBP1 showed that CaM removed the effect of CaBP1 at a molar ratio of ~a 1:1 in the cell. Alanine mutagenesis of the CaM binding site and deletion of the NT binding site of CaBP1 did not remove the effect of CaBP1 on the inactivation. Thus, NT does not appear to be the important anchoring site for the action CaBP1, and the role of the interaction of CaBP1 with the CaV1.2 NT remains to be determined.

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Structural Determinants of $Ca_V1.3$ L-Type Calcium Channel Gating

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$Ca_V1.3$ L-type Ca^{2+}-channels are key signal molecules for hearing, cardiac pacemaking and neuronal excitability. We have recently discovered a C-terminal intramolecular protein-interaction in $Ca_V1.3$ α_1-subunits as a gating modifier (CTM). This modulatory domain is absent in short C-terminal splice variants. Its presence shifts half maximal activation voltage ($V_{0.5}$) to more positive potentials and inhibits Ca^{2+}-dependent-inactivation (CDI). This regulation is present in long variants of human (Singh et al., 2008) and rat cDNA clones from pancreatic islets ($rCa_V1.3_{pan}$) but not in a clone derived from superior cervical ganglion ($rCa_V1.3_{scg}$). $rCa_V1.3_{scg}$ differs from $rCa_V1.3_{pan}$ at three amino acid (aa) positions (S244G, V1104A, A2073V), a polymethionine-stretch with two additional lysines, and one alternatively spliced locus (exon 31). We took advantage of these discrete differences to determine their role for the kinetic differences between $rCa_V1.3_{scg}$ and $rCa_V1.3_{pan}$ (in voltage-dependence of activation and CDI) by expressing channel mutants in tsA-201 cells and whole-cell patch-clamp-recordings.

Two aa (S244, A2073) in $rCa_V1.3_{scg}$ explained most of the functional differences to $rCa_V1.3_{pan}$. Mutation S244G even further enhanced CDI of $rCa_V1.3_{scg}$ and shifted its $V_{0.5}$ to more positive potentials. A2073V (located within the CTM) also shifted $V_{0.5}$ more positive but almost eliminated CDI. The cooperative action in the double-mutant restored gating properties (CDI, $V_{0.5}$) similar to $rCa_V1.3_{pan}$. Their effects are compatible with a recently proposed allosteric CDI mechanism implying CaM-mediated inhibition of the activation gate (Tadross and Yue, 2010). Analysis of inactivation kinetics suggest that G244 decreases the open probability (Po) of the inactivated gating mode, whereas A2073V reduces the maximal Po of $rCa_V1.3_{scg}$ as evident from increased gating currents.

G244 and V2073 affect CDI through different molecular mechanisms. Together they stabilize the gating behaviour of long $Ca_V1.3$ splice variants undergoing C-terminal-modulation.

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A Novel FRET-Based Assay Reveals 1:1 Stoichiometry of Apocalmodulin Binding Across Voltage-Gated Ca and Na Ion Channels

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Voltage-gated Ca and Na channels represent two important classes of ion channels where resident calmodulin (CaM) regulates channel gating, providing the cell with vital Ca^{2+} feedback. Functionally, a single CaM has been suggested to suffice for Ca^{2+}-modulation of channel gating (*Science*, **304**:432; JGP **131**:197). However, *in vitro* structural and biochemical studies have argued that multiple Ca^{2+}/CaMs may bind to the Ca_V channel carboxy-terminus (primary locus of CaM/channel interaction). Thus, the stoichiometry of apoCaM binding to the channel carboxy-terminus remains a crucial unknown. In this regard, live-cell FRET 2-hybrid assay has been used extensively to probe apoCaM binding. To gauge relative binding affinities, FRET efficiency between fluorophore-tagged apoCaM and a binding partner may be measured from acceptor-centric (3^3-FRET, *Neuron***39**:97) or donor-centric (E-FRET, *Biophys J***91**:L39) perspectives. Here, we exploit a fundamental asymmetry between these measures to estimate the stoichiometry of binding between apoCaM and channel carboxy termini: the ratio of maximal FRET efficiencies, as measured by 3^3-FRET and E-FRET methods, will approximate the ratio of donors to acceptors present in the complex. CFP and YFP concatemers with known ratios of donors and acceptors confirm this remarkable principle. Accordingly, we quantified both 3^3-FRET and E-FRET between CFP-apoCaM